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Genetic Editing of Herpes Simplex Virus 1 and Epstein-Barr Herpesvirus Genomes by Human APOBEC3 Cytidine Deaminases in Culture and *In Vivo*▽†

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Human APOBEC3 cytidine deaminases target and edit single-stranded DNA, which can be of viral, mitochondrial, or nuclear origin. Retrovirus genomes, such as human immunodeficiency virus (HIV) genomes deficient in the *vif* gene and the hepatitis B virus genome, are particularly vulnerable. The genomes of some DNA viruses, such as human papillomaviruses, can be edited *in vivo* and in transfection experiments. Accordingly, herpesviruses should be no exception. This is indeed the case for herpes simplex virus 1 (HSV-1) in tissue culture, where APOBEC3C (A3C) overexpression can reduce virus titers and the particle/PFU ratio ~10-fold. Nonetheless, A3A, A3G, and AICDA can edit what is presumably a small fraction of HSV genomes in an experimental setting without seriously impacting the viral titer. Hyperediting was found in HSV genomes recovered from 4/8 uncultured buccal lesions. The phenomenon is not restricted to HSV, since hyperedited Epstein-Barr virus (EBV) genomes were readily recovered from 4/5 established cell lines, indicating that episomes are vulnerable to editing. These findings suggest that the widely expressed A3C cytidine deaminase can function as a restriction factor for some human herpesviruses. That the *A3C* gene is not induced by type I interferons begs the question whether some herpesviruses encode A3C antagonists.

The seven-gene human *APOBEC3* (*A3*) cytidine deaminase locus came to the fore with the identification of APOBEC3G (A3G) as the interaction partner of the human immunodeficiency virus (HIV) Vif protein (8, 16, 26, 29, 30, 43, 57). These enzymes belong to a larger group that can edit nucleic acids, of which activation-induced cytidine deaminase (AICDA), responsible for class switch recombination and somatic hypermutation of rearranged immunoglobulin V region genes, is perhaps the most widely known (11). All functional A3 enzymes show specificity for single-stranded DNA (ssDNA). Since the reaction product is uridine (dU), A3 activity results in DNA peppered by C → U substitutions, referred to as hypermutants. Editing can range from a few cytidine targets to over 80% (2, 3, 8, 16, 26, 29, 30, 49, 54). To a good first approximation, all A3 enzymes preferentially edit ssDNA when the edited base is 5′ flanked by thymidine or cytidine, i.e., TpC and CpC. In contrast, AICDA prefers GpC and ApC (2, 3, 9, 17, 27, 39, 49, 54).

Since the HIV-encoded Vif protein protects its genome from the mutagenic effects of A3G, HIV hypermutants are associated with a defective or deleterious *vif* background (43). In contrast, hepatitis B virus (HBV) DNA is particularly susceptible to genetic editing by at least two A3 enzymes *in vivo* (54), perhaps because its small genome does not encode interferon or A3 antagonists. Double-stranded DNA (dsDNA) is prone to editing during replication or transcription when it is partially single-stranded. Human papillomavirus genomes are vulnerable to APOBEC3 editing *in vivo* and in transfection experiments (53). In contrast, vaccinia virus, which replicates in the cytoplasm, is apparently resistant to A3G (22).

Given their very large genomes, between 124 and 241 kb (32), herpesviruses, which replicate in the nucleus, might be particularly sensitive to A3 deamination, since even low levels of deamination, say, <0.1%, would introduce several hundred mutations per genome. The seven *A3* genes are expressed in a very wide variety of cell types, with some of the genes, notably human *APOBEC3A* and *APOBEC3G* (*A3A* and *A3G*), being strongly upregulated by type I interferons (IFNs) (5, 21, 40, 44, 55). Yet since herpes simplex virus (HSV) replication is comparatively resistant to IFN signaling and IFN-mediated responses in tissue culture (13, 34, 35, 41), they may not function as restriction factors. In contrast, *APOBEC3C* (*A3C*) is not only the most abundantly expressed of all *A3* genes across a wide range of tissues and cells but also is insensitive to IFN (19). It can edit transfected human papillomavirus (HPV) DNA and mitochondrial DNA (mtDNA) (46, 53). Hence, it is plausible that *A3C* has posed a particular problem for primate herpesviruses. Here it is shown that HSV-1 is particularly vulnerable to the editing effects of APOBEC3C both in tissue culture and *in vivo*. Equally, Epstein-Barr virus (EBV) genomes in EBV-transformed oligoclonal B-cell lines can be edited by at least one APOBEC3 enzyme.

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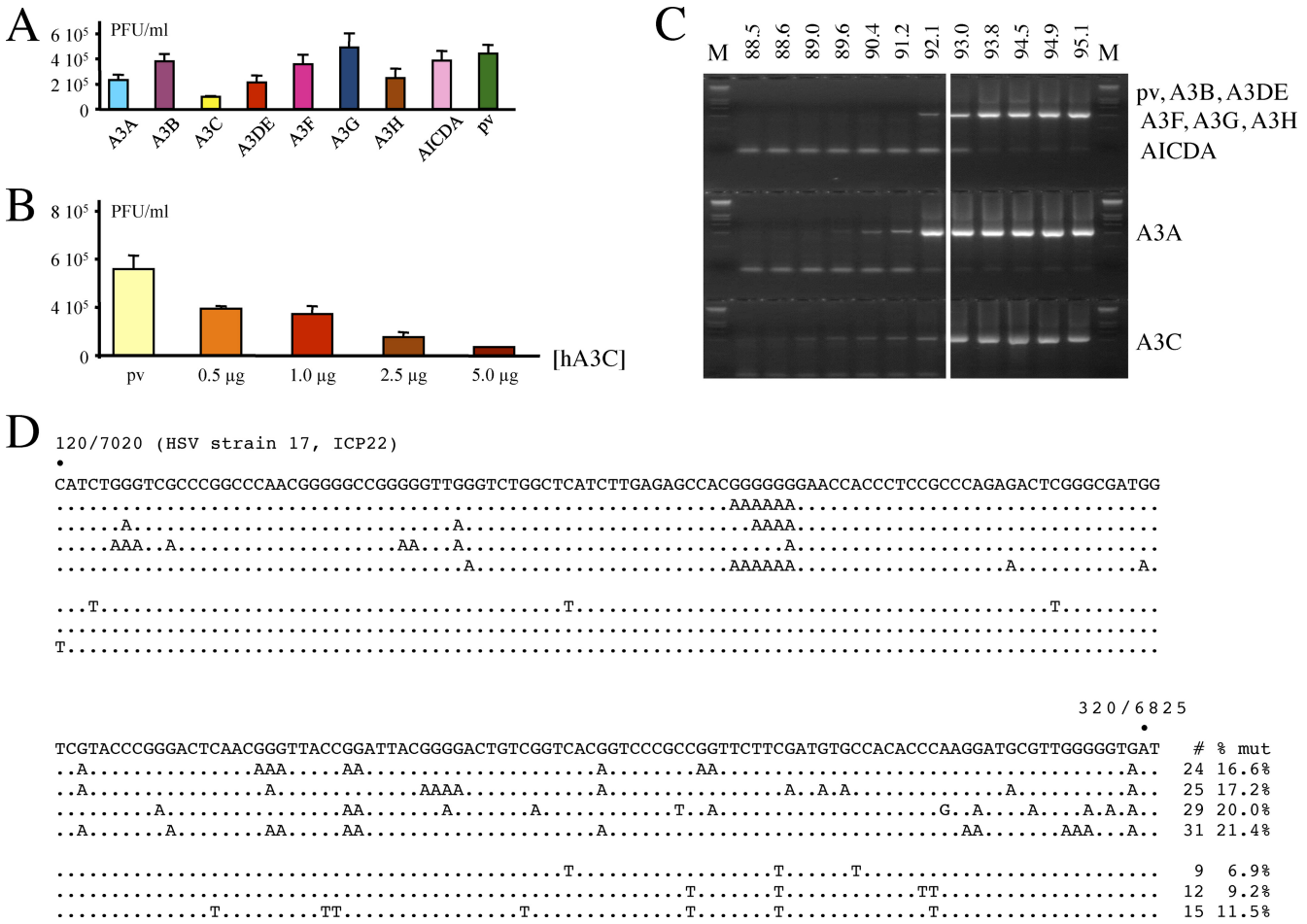

FIG. 1. Several human A3 enzymes may restrict HSV-1 replication and edit the genome. (A) Transfection of HeLa cells by 2.5 μg of plasmid DNA followed by HSV-1 infection. pv, empty vector. Titers are the means for triplicate experiments. (B) Dose-response relationship of supernatant titers as a function of A3C DNA concentration. The DNA concentration was maintained constant with the plasmid vector. (C) 3DPCR of HSV-1 DNA for all DNA transfections. The denaturation temperature (T_d) is given across the top; M, molecular weight markers. The HSV-1 sequence between the primers is 410 bp in length. The white vertical bar indicates the minimal T_d (93 to 93.8°C) for cloned wild-type HSV-1 DNA. Bands to lower temperature can be taken as *prima facie* evidence of cytidine deamination. (D) A selection of A3-edited HSV-1 genomes from the HeLa + pv transfection. For clarity, only 200 bp of the 410-bp *ICP22* sequence are shown, while differences are scored with respect to the reference sequence (G → A, n = 25, 10,250 bp; C → T, n = 9, 3,690 bp). The positions in the HSV-1 strain 17 sequence are also given. To the right of each sequence is the total number of C → T transitions on the plus or minus (shown as G → A transitions) strand.

MATERIALS AND METHODS

Cell culture and transfection. HeLa cells and Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (PAA) at 37°C in 5% CO_2. The EBV cell lines were maintained in RPMI supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (PAA). QT6 cells were maintained in Ham's medium supplemented with 100 U/ml penicillin, 2 mM glutamine, 5% tryptose phosphate, 1% chicken serum, and 10% fetal calf serum. HSV-1 strain 17 was grown in HeLa cells. Two days postinfection, supernatants were recovered and treated with 40 U of Turbo DNase (Ambion) for 30 min at 37°C and frozen at −80°C. Infections were carried out on 6×10^5 QT6 cells in 6-well plates at a multiplicity of infection (MOI) of 1. QT6 supernatants were treated with 40 U of Turbo DNase as described above.

For transfection, 5×10^4 HeLa cells were seeded in 24-well tissue culture plates and incubated for 24 h. Transfections were performed using Lipofectamine 2000 (Invitrogen) or jetPRIME. Briefly, HeLa cells were transfected with equal amounts (2.5 μg) of the individual expression plasmids in duplicate. Controls were performed in parallel without APOBECs. An enhanced green fluorescent protein (EGFP) expression plasmid was transfected in parallel, and the transfection efficiency was determined via flow cytometry: transfection efficiencies were ~70%. Transfection medium was changed after 3 h, and transfected cells were incubated for 24 h and infected with HSV-1 (MOI = 1) for 90 min. After 48 h of incubation, the virus-containing supernatants were collected after centrifugation and stored at −80°C. Western blotting was performed as described previously (46).

Plaque assay. Vero cells (7.5×10^4) were seeded in 24-well tissue culture plates and incubated for 24 h. The confluent cell monolayer was inoculated with serial virus dilutions in DMEM for 90 min and then overlaid with methylcellulose, incubated for 48 h, and washed with phosphate-buffered saline solution (PBS). Plaques were counted manually after fixing and staining with crystal violet.

Particle/PFU ratios. Particle counts of viral supernatants were determined by mixing the supernatants with a preparation of 250-nm-diameter biotin-conjugated latex beads (Sigma) of known concentration. The mixture was then adsorbed onto glass coverslips for 1 h at room temperature and fixed with methanol. Samples were labeled using the mouse VP5-specific monoclonal antibody (DM165) (31) to label capsids or the rabbit polyclonal PTNC antibody (37) to label whole virions and L particles. Secondary antibodies used were goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 568 conjugated antibodies (Molecular Probes). Latex beads were stained using Alexa 633-conjugated streptavidin (Molecular Probes). The relative number of virus particles was estimated according to the number of counted latex beads.

PCR. DNAs were extracted using an Epicentre kit. The HSV-1 *ICP22* gene primers were as follows: 5′ OUT, CGACGCGGGCCCGAGCRTATRCTY YAT; 3′ OUT, GGAAATGGCGGACACCTTCCTGGAYAYYAT; 5′ IN, CT CGTAGTAGACCCRAATCTCCACATT; 3′ IN, GCCGACGTACGCGATGA GATYAAT.

The outer and inner fragments were 880 and 461 bp, respectively. The first reaction involved standard amplification. Reaction parameters were as follows: 95°C for 7 min, followed by 42 cycles (each consisting of 95°C for 1 min, 60°C for 30 s, and 72°C for 3 min), and finally 20 min at 72°C. Differential amplification occurred in the second round (using 1 μl of the first-round reaction as input) by using an Eppendorf gradient Mastercycler S thermal cycler programmed to generate a 6°C gradient in the denaturation temperature. The reaction parameters were 89 to 95°C for 5 min, followed by 42 cycles (each consisting of 89 to 95°C for 1 min, 55°C for 30 s, and 72°C for 2 min), and finally 10 min at 72°C.

For HSV *ICP0*, the primers were as follows: 5′ OUT and IN, 5′ TTGCGCA ATTGCATCCARRTTTTCAT; 3′ OUT, 5′ GAGGGGGAACTCGTGGGTG YTGATT; 3′ IN, 5′ GGACAGCACGGACAYGGAAYTGTT.

The outer and inner fragments were, respectively, 420 bp and 217 bp without primers. The conditions were as for *ICP22*, except for a 63°C annealing step for first-round PCR and an 8°C gradient at the denaturation temperature. The differential DNA denaturation PCR (3DPCR) reaction parameters were 87 to 95°C for 5 min, followed by 42 cycles (each consisting of 87 to 95°C for 1 min, 63°C for 30 s, and 72°C for 2 min), and finally 10 min at 72°C.

For HSV *ICP8*, the primers were as follows: 5′ OUT, 5′ CAAAGCCCAAG ACGGCAACCACCATCAA; 3′ OUT, 5′ CTGGCTGGCTTCGAAGGCCGT GAAYGTA; 5′ IN, 5′ CACCTGGACCCCAGCACCCAGRCCCCAA; 3′ IN, 5′ GCTAAAATCCGGCATGAACAGCTGYAA.

The outer and inner fragments were, respectively, 810 bp and 263 bp without primers. The conditions were as for *ICP22* except for a 65°C annealing step for first-round PCR and an 8°C gradient in the denaturation temperature. The reaction parameters were 87 to 95°C for 5 min, followed by 42 cycles (each consisting of 87 to 95°C for 1 min, 62°C for 30 s, and 72°C for 2 min), and finally 10 min at 72°C.

The EBV *EBNA-1* gene primers were as follows: 5′ OUT, GTAGCATCTCT GTCTGGTGACCTTGAA; 3′ OUT, TTTTGGGGTCTCCGGACACCATCT CTA; 5′ IN, AGGCCTGGCTTGAGGCTCAGGACGCAA; 3′ IN, GACATG ATTCACACTAAAAGAGATCAA.

The outer and inner fragments were 567 and 254 bp, respectively. The first reaction involved standard amplification. The reaction parameters were 95°C for 7 min, followed by 42 cycles (each consisting of 95°C for 1 min, 60°C for 30 s, and 72°C for 3 min), and finally 20 min at 72°C. Differential amplification occurred in the second round (using 1 μl from the first-round reaction as input) by using a 10°C gradient in the denaturation temperature. The reaction parameters were 80 to 90°C for 5 min, followed by 42 cycles (each consisting of 80 to 90°C for 1 min, 60°C for 30 s, and 72°C for 2 min), and finally 10 min at 72°C.

The EBV *EBNA-2* gene primers were as follows: 5′ OUT, 5′ TAACGTGCA AGACGCTAAACTTAACCAA; 3′ OUT, 5′ AGCCTCGGTTGTGACAGAG GTGACAA; 5′ IN, 5′ TGTGTTTTGCTTTATCTGCCGCCATCA; 3′ IN, 5′ CGTCATATCCTAGCGGATCCCTATCAA.

The outer and inner fragments were, respectively, 907 bp and 345 bp without primers. The conditions were as for *EBNA-1* except for a 62°C annealing step for first-round PCR. For the 3DPCR, reaction parameters were 80 to 90°C for 5 min, followed by 42 cycles (each consisting of 80 to 90°C for 1 min, 63°C for 30 s, and 72°C for 2 min), and finally 10 min at 72°C. PCR products were cloned into the pCR2.1 Topo cloning vector (Invitrogen). Sequencing was outsourced to GATC.

RESULTS

A3C restricts HSV replication. To explore the hypothesis that A3 enzymes may impact HSV-1 replication, HeLa cells were transfected by a variety of human cytidine deaminases, including AICDA. Twenty-four hours posttransfection, the cells were infected with HSV-1 at a MOI of 1 and allowed to grow for a further 48 h, after which titers of virus supernatants were determined on Vero cells. A3C reduced HSV titers by ~4-fold (Fig. 1A), in keeping with transfection frequencies of ~70%. The other deaminases had no significant impact compared to controls. A titration was performed with increasing amounts of *A3C*, using a plasmid vector to provide for a constant DNA concentration. A dose-response relationship was obtained (Fig. 1B).

	Sample	mean titre PFU / ml	Complete particles /ml	Complete particles / PFU
A	HeLa mock	$8.4\ 10^6$	$1.2\ 10^8$	14
	HeLa + vector	$4.2\ 10^6$	$4.7\ 10^7$	11
	HeLa + A3C	$2.8\ 10^5$	$3.4\ 10^7$	121
	HeLa + A3G	$1.3\ 10^6$	$3.6\ 10^7$	28
B	HeLa + vector	$6.0\ 10^6$	$1.2\ 10^8$	20
	HeLa + A3C*	$1.4\ 10^6$	$3.3\ 10^7$	24
C	HeLa + si control	$3.2\ 10^7$	$1.9\ 10^8$	6
	HeLa + siA3C	$2.9\ 10^7$	$6.6\ 10^7$	2

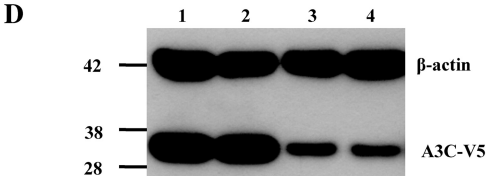

FIG. 2. A3C levels can impact particle/PFU ratios for HSV-1. (A) Impact of active *A3* constructs. (B) Impact of the *A3C* C957S inactive mutant. (C) Impact of A3C siRNA. (D) Western blot of V5-tagged A3C and β-actin loading control for uninfected HeLa cells at 48 h; lanes 1 and 2, 400 ng A3C-V5 tag plus 1 μg siRNA control; lanes 3 and 4, 400 ng A3C-V5 tag plus 1 μg siA3C RNA. Molecular mass markers (in kDa) are shown to the left.

Another measure of HSV infectivity is the particle/PFU ratio, which is typically of the order of 10 to 100 for herpesviruses (12). When HSV was grown on *A3C*-transfected HeLa cells the ratio was increased ~10-fold (Fig. 2A), indicating a higher number of defective particles in this population. In contrast, A3G failed to seriously impact the ratio. The vector transfection control yielded a titer comparable to that of virus from mock-transfected HeLa cells. An A3C catalytic mutant (C97S) yielded a particle/PFU ratio comparable to that of the plasmid vector control (Fig. 2B), while transfection of a small interfering RNA (siRNA) to A3C lowered the particle/PFU ratio compared to that of the siRNA control (Fig. 2C). Since the majority of cells were lysed at 48 h, the efficiency of siRNA knockdown was determined with uninfected cells. At 48 h, there was substantial knockdown of A3C-V5 as shown by Western blotting (Fig. 2D).

Total DNA was extracted and analyzed by 3DPCR, which is a derivative of PCR that allows selective amplification of AT-rich DNA amid excess normal DNA (48). It exploits the lower denaturation temperature (T_d) of AT-rich DNA by carrying out PCR with a T_d gradient. If the lowest positive T_d at which DNA is recovered is lower than that of the control, this can be considered *prima facie* evidence of recovery of AT-rich variants. Since the product of APOBEC3 editing of ssDNA is uridine, which base pairs as thymidine and is readily copied by

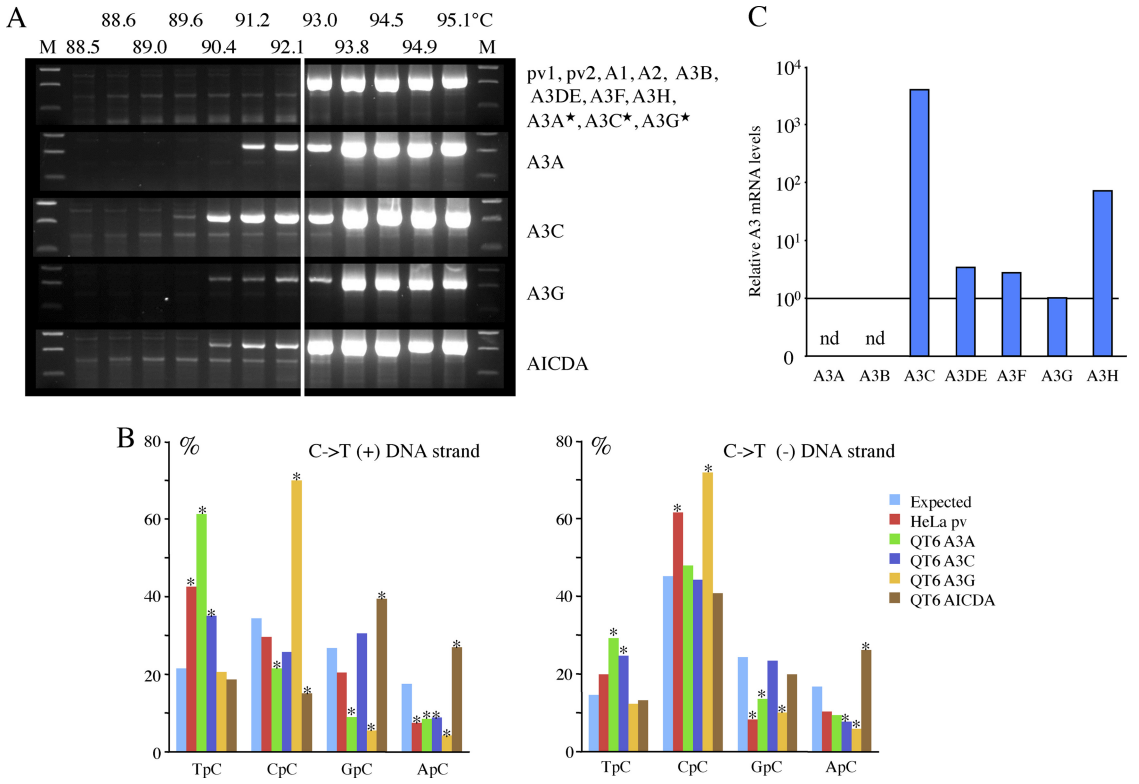

FIG. 3. Four human cytidine deaminases can hyperedit HSV-1 genomes. (A) Agarose gels of *ICP22* 3DPCR DNA products from HSV-1-infected quail QT6 cells expressing various *A3* genes. The annotation is as for Fig. 1C. Stars indicate A3A, A3C, and A3G catalytic mutants. (B) 5′ dinucleotide context associated with editing on the plus and minus strands. Asterisks denote significant deviations from the expected values (χ^2 test, $P < 0.05$) (QT6 + A3A, G → A, $n = 24$, 9,840 bp; C → T, $n = 31$, 12,710 bp; QT6 + A3C, G → A, $n = 50$, 20,500 bp; C → T, $n = 78$, 31,980 bp; QT6 + A3G, G → A, $n = 32$, 13,120 bp; C → T, $n = 8$, 3,280 bp; QT6 + AID, G → A, $n = 9$, 3,690 bp; C → T, $n = 24$, 9,840 bp). (C) TaqMan transcriptome analysis of the seven *A3* genes from HeLa cells. The levels have been normalized to those of *A3G* (horizontal bar). nd, not detected.

Taq polymerase, it is understandable that 3DPCR has proven immensely useful in analyzing A3 editing (5, 36, 38, 44, 46–48, 51, 53, 54).

We chose the *ICP22* immediate-early gene, which encodes a transcription factor involved in regulation of the viral cycle. As can be seen from Fig. 1C, HSV-1 DNA was recovered at denaturation temperatures down to 88.5°C for A3C and 89.6°C for A3A. Since the lowest denaturation temperature for cloned reference HSV-1 is ~93 to 93.8°C (indicated by a white vertical line in Fig. 1C), recovery of HSV-1 DNA at lower temperatures is evidence of AT-rich variants. However, hyperedited HSV-1 DNA was obtained even for the HeLa plasmid vector (pv) transfection control (Fig. 1C). A sequence analysis of 3DPCR products derived from the pv sample with a T_d of 92.1°C revealed hyperedited HSV genomes (Fig. 1D), with both DNA strands being vulnerable. The mean level of editing was 13% per clone, with a range of 1 to 23% ($n = 25$).

To overcome this endogenous editing background from HeLa cells, HSV-1 was passaged four times on the quail QT6 cell line (HSV-1/QT6). The avian lineage does not encode any *A3* genes and has been shown not to give an endogenous editing background when stocks of HIV or HBV are made following transfection (17, 38, 54). DNase treatment of the supernatant was performed each time in order to reduce passive transfer of contaminating edited DNA. As expected, HSV-1/QT6 stock virus failed to give a background signal for editing (Fig. 3A, top). Accordingly, QT6 cells were transfected by human cytidine deaminases and subsequently infected by HSV-1/QT6 virus for 48 h.

When total cell DNAs were examined by 3DPCR, AT-rich DNA was identified from the AICDA, A3A, A3C, and A3G transfections but not from the others (Fig. 3A). Not surprisingly, three A3 catalytic site mutants (A3A C105S, A3C C97S, and A3G C281S) failed to edit HSV-1 genomes. Judging by the lowest denaturation temperature (89.6°C) and band intensities, A3C impacted the HSV-1 genome more severely than the others (Fig. 3A). Sequence analysis of cloned 3DPCR products revealed extensive editing, with mean mutation frequencies of ~23% for the plus strand and between 23 and 42% for the minus strand. For A3A, A3C, and A3G, the minus strand was systematically more heavily edited than the plus strand, whereas for AICDA, the means were comparable (Table 1). Dinucleotide context analysis showed that editing was biased in favor of TpC for A3A and A3C, CpC for A3G, and GpC and ApC for AICDA (Fig. 3B), all of which have been previously noted for other virus genomes (2, 3, 7, 10, 14, 17, 28, 45, 51–54, 56). There was good concordance between the editing biases on both HSV-1 DNA strands (Fig. 3B). With these reference sets, the hyperedited sequences derived from the HeLa stock virus could be examined (Fig. 3B). While the overlap wasn't

TABLE 1. Essential statistics for HSV-1 and EBV edited genomes

Virus (T_d, °C)	Deaminase, cell line, and/or sample	Hyperediting	No. of sequences	Mean mut./seq.[a]	% GC → AT	% other
HSV-1 (91.1–92.1)	A3A/QT6	C → T	31	30	93.7	6.3
		G → A	24	61	96.7	3.3
	A3C/QT6	C → T	78	32	96.2	3.8
		G → A	50	46	96.9	3.1
	A3G/QT6	C → T	8	32	95.1	4.9
		G → A	32	56	96.4	3.6
	AICDA/QT6	C → T	24	32	95.6	4.4
		G → A	10	34	92.9	7.1
HSV-1 (92.1)	HeLa	C → T	9	14	93.4	6.6
		G → A	26	22	94.5	5.5
HSV-1 (90.4–92.1)	P9	C → T	21	25	91.2	8.8
	P9	G → A	94	36	96.3	3.7
HSV-1 (93.8–94.9)	P1		39	2	36.1	63.9
	P5		250	3	64.7	35.3
	P6		186	2	64.2	35.8
	P8		29	4	64.2	35.8
	P9		134	2	50.2	49.8
	P11		30	3	29.8	70.2
	P13		29	5	70.5	29.5
	P14		28	6	77.6	22.4
EBV (82.5–89.8)	EBV-blast P1	G → A	54	17.5	96.8	3.2
	EBV-blast P2	G → A	31	21.6	97.9	2.1
	EBV-blast P3	G → A	32	22.1	97.8	2.2
	EBV-blast Z	G → A	99	19.2	97.5	2.5

[a] Mean number of mutations per sequence.

perfect, there was similarity with the patterns for both A3A and A3C editing. A TaqMan transcriptome analysis (40) of the 7 *A3* genes in HeLa cells was performed. Since *A3A* levels were not detected while *A3C* levels were far higher than those for any other *A3* gene (Fig. 3C), it is most likely that HSV-1 editing is due predominantly to A3C. To establish an overall hypermutant editing frequency, we performed a limiting dilution (cf. reference 47) of the first-round products, followed by PCR at 95°C and in-parallel 3DPCR at 92.1°C. This yielded a differential hypermutant frequency of $\sim 10^{-3}$. Since 3DPCR tends to underestimate lightly edited DNA, this represents an underestimation (54).

Is A3 editing of the HSV-1 genome physiologically relevant? To address this question, we analyzed total DNA extracted directly from several HSV-1-associated lesions, notably pharyngeal washes and labial swabs. Fourteen samples were analyzed, the underlying pathologies ranging from prior liver transplantation to cancer. Using a nested PCR/3DPCR approach, eight samples scored positive for HSV-1 *ICP22* DNA. The results were variable, with the last denaturation temperature ranging from 89.6 to 93.8°C (Fig. 4A). Since the lowest T_d for cloned HSV-1 from QT6 cells is ~93 to 93.8°C, samples P5, P6, and P9 apparently include hyperedited HSV-1 genomes. Sequencing of cloned 3DPCR DNA revealed extensively hypermutated sequences for P5 and P9, although only for P9 was an extensive group of hypermutated sequences recovered, with up to 52 targets edited on both strands (Fig. 4B). Dinucleotide contexts associated with P9 editing, from greatest to least association, were TpC, CpC, and RpC (Fig. 4C), typical of editing by A3 deaminases and highly comparable to that observed for HeLa derived stock (Fig. 3B). Limiting dilution of first-round products for patient 9 yielded a hypermutant frequency of $\sim 10^{-4}$.

To see if there were lightly edited HSV-1 sequences, 3DPCR products in the 93 to 94.5°C temperature range were cloned and sequenced. Numerous sequences encoded 1 to 10 transitions (Fig. 4D). Those with 1 to 4 C → T transitions showed no dinucleotide bias and probably reflect the AT-rich end of the HSV mutant spectrum (Fig. 4C). Furthermore, since the dominant PCR-related mutations are AT → GC, the majority cannot be ascribed to PCR error (15, 33). In contrast, those with 5 or more monotonous C → T transitions in either strand showed the same dinucleotide biases as hypermutated genomes from P9 (Fig. 4C), indicating that they do indeed reflect A3 editing. Accordingly, A3-edited HSV genomes were recovered from 4/8 samples (P5, P6, P9, and P14), albeit to different degrees. Finally, to ascertain whether other regions of the HSV-1 genome could be edited *in vivo*, the *ICP0* and *ICP8* genes were analyzed from patient 5 and 9 DNA, respectively. Hypermutated sequences were readily recovered (see Fig. S1A and B in the supplemental material), indicating that most probably all parts of the HSV-1 genome are vulnerable to editing.

Since hypermutated HSV genomes are physiologically relevant, we were interested in whether other herpesvirus genomes were also susceptible to A3 editing. Since the A3C gene is highly expressed in leukocytes (40, 46), we tested whether Epstein-Barr virus (EBV) genomes from transformed peripheral blood mononuclear cell lines were vulnerable. In such cell lines, EBV is found in its latent form with little transcription,

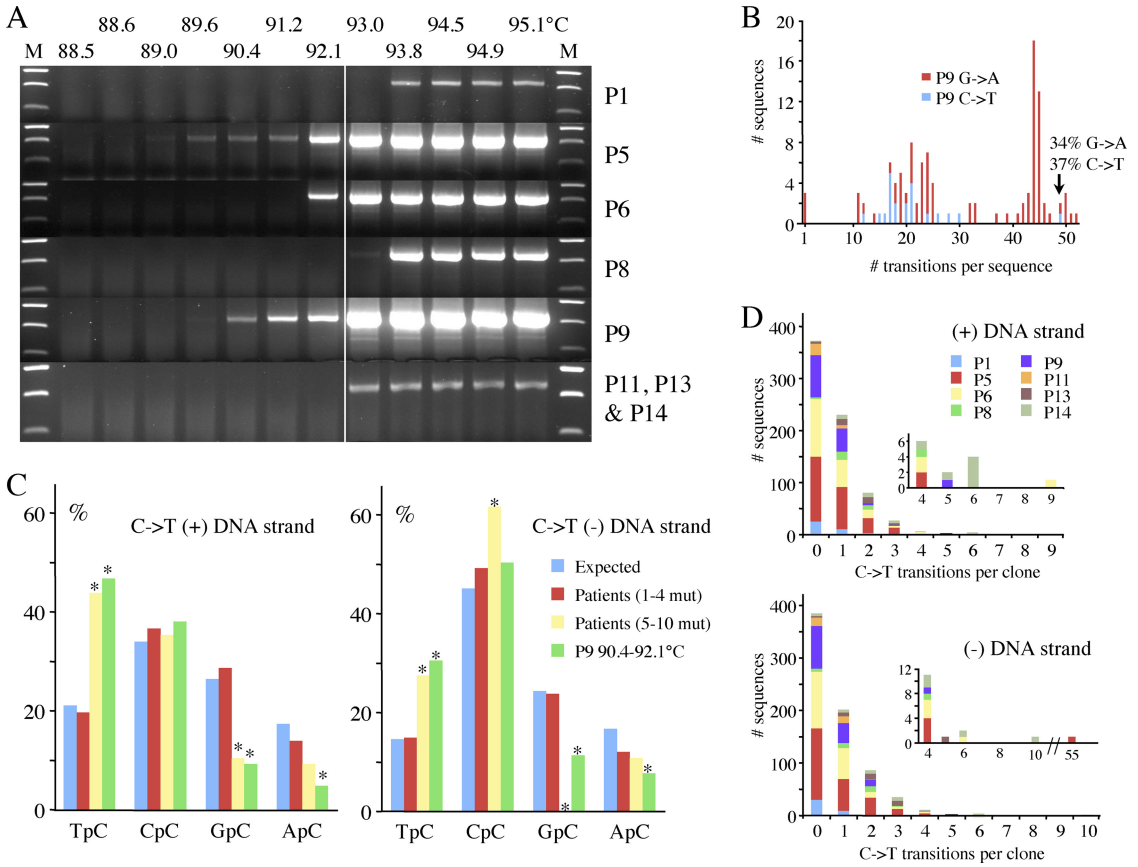

FIG. 4. HSV-1 genomes can be edited *in vivo*. (A) Agarose gels of *ICP22* 3DPCR DNA products from total DNA derived from pharyngeal washes or labial swabs. The annotation is as for Fig. 1C. (B) Frequency distribution of A3-edited HSV-1 sequences from patient P9. Since the cytidine composition of the two strands varies (32% plus strand; 35% minus strand), the % editing differs slightly (P9 G → A, $n = 88$, 36,080 bp; C → T, $n = 21$, 8,610 bp). (C) 5′ dinucleotide context associated with editing on the plus and minus strands for the collection of sequences from patient P9 ($T_d = 90.4$ to 92.1°C). Asterisks denote significant deviations form the expected values (χ^2 test, $P < 0.05$). Also shown are sequences derived at a higher T_d (93.8 to 94.5°C) with 1 to 4 C → T transitions or 5 to 10 transitions pooled from all patients [Patients (1–4 mut.), $n = 713$, 292,330 bp; Patients (5–10 mut.), $n = 10$, 4,100 bp; P9, G → A, $n = 94$, 38,540 bp; C → T, $n = 21$, 8,610 bp]. (D) Frequency distribution of sequences derived at 93.8 to 94.5°C from all eight patients for both strands. The insets expand the region covering 4 to 55 bp.

although EBNA-1 is transcribed (18). Using a nested PCR/3DPCR approach, we amplified part of the *EBNA-1* gene from total DNA. Four of five EBV$^+$ cell lines (P1, P2, P3, and Z) (Fig. 5A) proved positive for edited EBV DNA given that the reference denaturation temperature for the segment is 86.7°C, far lower than that for HSV-1, reflecting a lower GC content (EBV *EBNA-1*, 54%; HSV-1 *ICP22*, 67%). Cloning and sequencing of the 3DPCR products revealed extensive cytidine editing (Fig. 5B), ranging from 10 to 53% (Fig. 5C). To ascertain whether other regions of the EBV genome could be edited *in vivo*, the *EBNA-2* gene from patient Z was analyzed. Hypermutated sequences were readily recovered (see Fig. S1C in the supplemental material), indicating that most probably all parts of the EBV genome are vulnerable to editing.

The dinucleotide context associated with editing, in order of greatest to least, was CpC, TpC, and RpC, typical of A3G editing (Fig. 5D). A PCR/3DPCR limiting dilution of analysis of Z DNA yielded a hypermutant frequency of $\sim 10^{-3}$ (95°C versus 85.3°C). An APOBEC3 transcriptome analysis of these cell lines showed that *A3C* was the most abundantly expressed gene, almost a log more than *A3G*, with *A3A* being the least expressed (46).

DISCUSSION

Like other viral DNA genomes, those of some human herpesviruses are vulnerable to APOBEC3 editing. For HSV-1, A3C appears to be an important restriction factor and can impact both the titer and particle/PFU ratio. The relevance of the experimental findings is confirmed by the recovery of A3-edited genomes in uncultured samples (Fig. 4). HSV-1 is relatively insensitive to type I interferons, which should help protect it from the A3 enzymes whose genes are upregulated by them. However, since *A3C* expression is essentially insensitive to alpha IFN (IFN-α), the present findings raise the question as to whether HSV-1 encodes an A3C antagonist, given that it is sensitive to overexpression of *A3C* (Fig. 1A and B), or whether productive replication occurs in *A3C*^{neg} or *A3C*^{low} cells. Certainly, *A3C* siRNA knockdown shows that replication is sensitive to

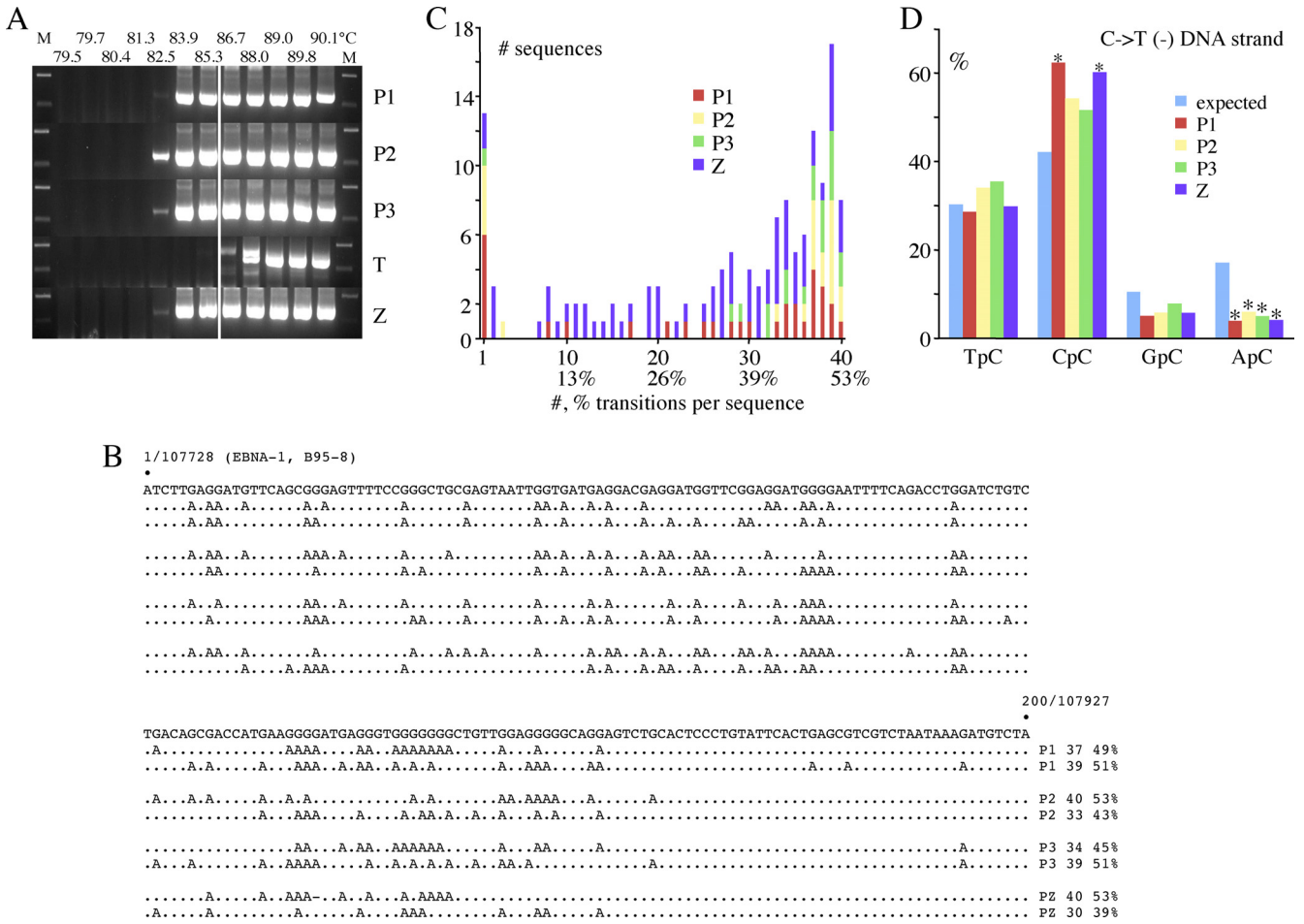

FIG. 5. Hyperediting of EBV genomes in EBV-immortalized human B cell lines. (A) Agarose gels of EBNA-1 3DPCR DNA products from total DNA derived from five EBV-immortalized human B cell lines. The annotation is as for Fig. 1C. The P1 to -3 lines are all *ung*$^{-/-}$; Z is *AICDA*$^{-/-}$, while T is an EBV-cell line from a normal patient without any known leukocyte defect. (B) A selection of hyperedited sequences from P1 to -3 and Z. To the right are the number and % of cytidine residues edited. Plus-strand hypermutants were not always recovered and hence were excluded from analyses. (C) Frequency distribution of A3-edited EBV sequences from four EBV-transformed cell lines. Only minus-strand editing was identified (P1, $n = 32$, 6,400 bp; P2, $n = 21$, 4,200 bp; P3, $n = 19$, 3,800 bp; Z, $n = 72$, 14,400 bp). (D) 5′ dinucleotide context associated with minus-strand editing. Asterisks denote significant deviations from the expected values (χ^2 test, $P < 0.05$).

the expression levels in HeLa cells (Fig. 2C). In short, does HSV editing *in vivo* reflect A3 restriction of genomes defective for the antagonist, analogous to HIV/simian immunodeficiency virus (SIV) hypermutants on a Δ*vif* background (4, 8, 16, 26, 29, 30, 42, 43, 57), or replication in *A3C*$^{neg/low}$ cells, analogous to HBV infection in cirrhosis, since this virus does not encode any known IFN-α or A3 antagonist (54)? With this in mind, it would be particularly interesting to determine A3C expression in sensory neurons, particularly since *A3* levels are generally low in uninfected brain (40). However, since B cells express high levels of *A3* genes yet are propitious for EBV replication, simple correlations with bulk transcription levels may not be reliable. Given that A3C is expressed in a wide range of different cells and tissues and is invariably the most highly expressed of all the seven human *A3* genes, variability in expression levels might perhaps account for part of the variability in plaque titers and particle/PFU ratios as a function of different cell lines. For example, varicella-zoster virus grown on a human melanoma cell line shows one of the highest particle/PFU ratios for any virus (6). However, for herpesviruses other factors can impinge on the particle/PFU ratio (1).

Highly edited genomes are clearly defective. Indeed, if A3 editing of *ICP22* is representative of the whole genome, ~40% editing of cytidine targets in the plus or minus strands translates into >20,000 C → T transitions per genome. Even lightly edited *ICP22* DNA with 5 edits/410 bp translates into ~1,860 substitutions per genome, which is far above the average mutation rate for DNA genomes, and so these too must be defective. Indeed, even a log lower amount of editing would also result in defective genomes. Presumably the vast majority of A3-edited HSV-1 genomes must be defective. Given this and a 68.3% GC content (20), it is unlikely that A3 editing contributes to HSV evolution. In a comparison of HSV-1 genomes, GC → AT transitions slightly outnumbered AT → GC transitions (50). However, when normalized to base composition, AT → GC transitions were more frequent, which again suggests that A3C activity

does not impact HSV genome evolution. A3C presumably accesses replicating HSV DNA in the nucleus. That it impacts the particle/PFU ratio suggests that the lesions are in the packaged genomes, whether as dG-dU base pairs or as incorrectly repaired derivatives.

An interesting feature of the A3 editing described here is that while both strands are edited, we invariably recover more minus-strand than plus-strand hypermutants (G → A as opposed to C → T) (Table 1). Furthermore, the degree of editing was usually greater for the minus-strand than for the plus-strand hypermutants. This feature also shows up for mtDNA and nuclear DNA editing and for the A3-edited HPV hypermutants (46, 53). The variety among such genomes and amplification primers suggests that it is not an artifact but reflects some feature associated with A3 editing. As the minus strand is transcribed, some degree of protection by the transcription complex is clearly irrelevant.

Since A3 editing of the herpesvirus genomes occurs for HSV-1 and EBV genomes in their lytic and latent forms, respectively, it is possible that some of the other six human herpesviruses may be vulnerable too. The three EBV cell lines P1, P2, and P3 were deficient in the human uracil *N*-glycosylase (UNG), which we have shown to be an important component in the dynamics of A3-initiated catabolism of DNA: suppression of UNG activity resulted in higher frequencies of edited DNA (46). Yet in the present context, UNG was not crucial to detection of A3-edited EBV genomes since recovery of such genomes from the EBV$^+$ Z cell line (*ung*$^+$ yet *AICDA*$^{-/-}$) was comparable, while the EBV$^+$ line "T," an EBV cell line from a donor without any known genetic defect, failed to yield hyperedited EBV DNA (Fig. 5A). Both HSV-1 and EBV encode divergent UNG enzymes that are able to excise uridine from DNA and are found at the replication fork. They are clearly orthologous to mammalian UNGs (23). Whether these viral UNG enzymes have any impact on detection of A3-edited genomes needs to be established.

The *A3* gene locus appears with the emergence of placental mammals, and while it is generally bounded by the *cbx6* and *cbx7* genes, there is considerable variation (24). For example, the rat and mouse genomes encode a single *A3* gene, the cow genome two, cats three/four, and horses five/six, while the primate lineage encodes seven *A3* genes, six of which are functional (24, 25). Since many of these genes are phylogenically grouped with *A3C*, it is possible that they could represent yet another cross-species barrier, particularly in the sense of herpesvirus transmission to an animal with a more complex *A3* locus. Since herpesviruses may go back 300 million to 400 million years, while the initial single gene *A3* locus arose ~125 million years ago, cytidine deamination would seem to be a relatively recent restriction factor.

In conclusion, at least two human herpesviruses are vulnerable to A3 editing, a physiologically relevant observation. The findings extend the list of genomes which are vulnerable: retroviral (3, 10, 14, 16, 26, 28–30, 43), adeno-associated (7), human papillomavirus (53), human mitochondrial, and nuclear genomes (46), as well as transfected plasmid DNA (44). It appears a plausible working hypothesis that yet more DNA viruses are restricted by A3 enzymes.

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